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Original Article

ARBUSCULAR MYCORRHIZAL FUNGI ASSOCIATED WITH TEA GROWING UNDER TWO DIFFERENT AGRICULTURAL INTENSITIES

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ABSTRACT

Arbuscular mycorrhizal fungi (AMF) associated with the rhizosphere of tea [Camellia sinensis (L.) O. Kuntze] were studied in an untreated site (UTS) and a treated site (TS). UTS was a bare land where addition of fertilizers and other common agricultural practices have never been practised, whereas in TS chemical fertilizers as well as other pesticides have been generally applied in tea plantation. The study revealed overall 50 AMF morphotypes belong to fourteen genera, viz., Acaulospora, Ambispora, Claroideoglomus, Corymbiglomus, Dentiscutata, Dominikia, Funneliformis, Gigaspora, Glomus, Racocetra, Rhizophagus, Sclerocystis, Scutellospora and Septoglomus. The present study clearly indicated that tea rhizosphere harboured a plethora of AMF species. AMF being important components of agroecosystems, a detailed study of AMF adapted to the confined environment may be a pre-requisite to select the suitable AMF inoculum for field utilization.

KEYWORDS: Arbuscular Mycorrhizal Fungi (AMF), Camellia Sinensis, Colonization, Diversity & Dark Septate Endophyte (DSE)

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INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) comprise the most common mycorrhizal association and form mutualistic relationships with over 80% of all vascular plants (Brundrett, 2002). The association is characterized by the movement of plant-produced carbon to the fungus and fungal-acquired nutrients to the plant (Read et al. 1992). AMF are obligate mutualists belonging to the phylum Glomeromycota and have a ubiquitous distribution in global ecosystems (Redecker et al. 2000). AMF are the most important microbial symbioses for the majority of plants and influence plant community development under conditions of P-limitation. They facilitate nutrient uptake, improve water relations and above-ground productivity. They also act as bioprotectants against pathogens and toxic stresses. As obligate symbiont, AMF are believed to be dependent upon the host plant for fixed carbon. The plant receives a variety of benefits which may result in increased growth, improved water relations (Davies et al. 1993), pest and disease resistance (Hooker et al. 1994), enhanced nutrient uptake over non-mycorrhizal plants (George et al. 1995), and modification of root morphology (Berta et al. 1990). The most important of these benefits is increased nutrient uptake, notably of immobile nutrients such as P and Zn (Bolan, 1991; Bürkert and Robson, 1994). Phosphorus concentration had been shown to increase up to four times in mycorrhizal plants (Karagiannidis and Hadjisavva-Zinoviadi, 1998). Some plant roots are also colonized by a diverse group of thick-walled, pigmented and septate fungi called dark septate endophyte (DSE). DSE facilitated nutrient uptake of the host plant, alterations in host water uptake, stress tolerance and utilization of wider nutrient pools by the host

(Mandyam and Jumpponen, 2005).

Tea [*Camellia sinensis* (L.) O. Kuntze], which belongs to the family Theaceae is a widely consumed non-alcoholic beverage and one of the most important cash crops of Assam. Tea cultivation plays a major role in the economy of this region. In tea plantations, agrochemicals are extensively used for managing insects, pests, diseases and weeds. These agrochemicals can be toxic to the non-target organisms. However, exploitation of soil microorganisms offers an attractive alternative to the use of harmful chemicals. AMF are considered as one of the important components of agro-ecosystems and hence a detailed study on diversity of AMF adapted to the local environment may be a pre-requisite to select the best AMF inoculum for field utilization. Studies on AMF diversity associated with tea grown in natural and cultivated ecosites have been conducted in other parts of India, such as in the Kumaun region of Uttaranchal Himalaya, India (Singh et al. 2008). Although *C. sinensis* has been extensively cultivated in many parts of Assam, the detailed studies on diversity of AMF associated with *C. sinensis* of Udalguri district in particular have so far not been conducted.

Therefore, in the present study, we focused on the study of diversity of AMF in the rhizosphere of *C. sinensis* cultivated under two different agricultural intensities, where one site is a bare land wherein no prior agricultural practices were performed, considered as untreated site (UTS). The other site was considered as treated site (TS), which is an established tea garden, where fertilizers are routinely applied to increase the yield of tea. Large amounts of insecticides, fungicides, herbicides and acaricides are also used in treated site as *C. sinensis* is highly susceptible to pests and diseases.

MATERIALS AND METHODS

Sampling

Root samples of *C. sinensis* and rhizosphere soil samples were collected at seasonal intervals from September 2013 to August 2014 from both treated site (TS) and untreated site (UTS) of Udalguri district (105.16 m above MSL, 26.7452° N, 92.0962° E), Assam, India. The study site experiences four distinct seasons, the Winter (December to February), the Pre-monsoon (March to May), the Monsoon (June to August) and the Retreating Monsoon (September to November) [*Source:* Forest Department, Udalguri district]. Root samples as well as rhizosphere soil samples were collected at the commencement and cessation of each season. The average annual rainfall of this area is about 2,000 mm and has a temperature range of 13.50º C to 34.50° C. Relative humidity ranges between 82% and 88%. UTS was a bare land where addition of fertilizers and other common agricultural practices have never been practised, whereas in TS chemical fertilizers as well as other pesticides have been generally applied in tea plantation. Root samples were collected by excavating the whole plant. Roots of ten plants were randomly selected from each site. The roots were gently washed with water and fixed in FAA (formalin: glacial acetic acid : 70% ethyl alcohol 5 : 5 : 90, v : v : v). Soil samples were collected in sterilized plastic bags. Roots as well as the soil samples were brought to the laboratory for further analysis.

Root Staining and Assessment of Fungal Colonization

The fixed roots were washed free of the FAA and cut into approximately 1 cm segments. The roots were then cleared in 10% (w/v) KOH by heating at 90°C for 1 to 2 hours, depending on the degree of hardness and lignifications of the roots. Post-clearing bleaching was done with alkaline hydrogen peroxide (0.5% NH4OH and 0.5% H2O2 v/v in distilled water). Roots were rinsed with distilled water, treated with 1% HCl and stained with 0.05% w/v trypan blue (Phillips and Hayman, 1970). The stained root samples were mounted on microscope slides in lacto glycerol and examined for AMF and DSE structures under light microscope. The percentage of root length colonized by AMF and DSE was

estimated according to the magnified intersection method (McGonigle et al. 1990).

Isolation, Enumeration and Identification of Spores

Rhizosphere soils of ten *C. sinensis* plants were collected from each sampling site. The spores were extracted from 25g soil sample. AMF spores from the soil sample were isolated and extracted by wet-sieving and decanting method of Gerdemann and Nicolson (1963). After wet-sieving and decanting through a series of 710 to 37µm sieves, the residues on the sieves were washed into beakers. The beaker contents were filtered through a filter paper, which was then spread on a petri dish. Spores were counted using a dissection microscope at 40X magnification. Sporocarps and spore clusters were considered as one unit. The isolated AMF spores were picked up using a needle and mounted in polyvinyl alcohol-lactoglycerol: Meltzer's reagent (1:1, v:v) for identification. Taxonomic identification of spores was based on morphological descriptions published by International Culture Collection of Vesicular and Arbuscular Mycorrhizal Fungi (http://invam.caf.wvu.edu), AMF phylogeny (www.amf-phylogeny.com) and Oehl and Sieverding (2004).

Analysis of Soil Physicochemical Properties

Soil texture was determined using sodium hexametaphosphate method (Allen et al. 1974). For determining soil moisture content, 10g of freshly collected soil was oven dried and weight was determined. Soil pH was determined using a digital pH meter. Available soil phosphorus was determined following molybdenum blue method (Allen et al. 1974). The soil organic carbon was estimated using colorimetric method (Anderson and Ingram, 1993).

Data Analysis

Spore density was expressed as total number of AMF spores occurring in 25 g of soil sample. Species richness was expressed as numbers of AMF species in 25g soil sample. Relative abundance, isolation frequency, Shannon-Wiener index of diversity (H') and Simpson's index of dominance (D) were calculated as outlined by Dandan and Zhiwei (2007). Standard errors of means were calculated. Analysis of variance (ANOVA) was used to assess the significance of variance on AMF and DSE colonization between the two study sites. The relationships among mycorrhizal structural colonization and soil physico-chemical properties were analyzed by Pearson's correlation coefficient values.

RESULTS AND DISCUSSIONS

The soil physicochemical characteristics are presented in Table 1. Soils were determined to be sandy loamy in texture. pH of the soils were moderately acidic. Soil organic carbon content as well as available phosphorus were higher in TS as compared to that of UTS. Roots of *C. sinensis* from both TS and UTS had dual colonization of AMF and DSE. DSE colonization was characterized by parallel light brown septate hyphae and intracellular microsclerotia formation. Roots samples were found to be fairly colonized by AMF in both the sites throughout the year. AMF structures, i.e. arbuscules, vesicles, and intercellular hyphae were present in all the root samples. In TS the percentage of total root length with AMF colonization ranged from 20.23% to 76.85%, while in UTS it ranged from 32.45% to 88.67%. While the lowest AMF colonization was observed in September, December had the highest colonization in both the sites (Figure 1). The percentage of root length with DSE colonization ranged from 0.78 (TS) to 8.49 (UTS). TS showed an AMF spore range of 352 to 985, while in UTS spore number ranged from 726 to 1380 (Figure 2). Overall 50 AMF morphotypes belonging to fourteen genera, viz., (*Acaulospora, Ambispora, Claroideoglomus, Corymbiglomus, Dentiscutata, Dominikia, Funneliformis, Gigaspora, Glomus, Racocetra, Rhizophagus, Sclerocystis, Scutellospora* and *Septoglomus*) were isolated

from *C. sinensis* rhizosphere soil. Out of these 44 species were identified from TS, whereas 48 species were identified from UTS.

ANOVA showed no significant variation ($P < 0.05$) between AMF colonization in TS and UTS. DSE colonization of TS and UTS showed significant variation ($P < 0.05$). AMF colonization was significantly higher ($P < 0.05$) than DSE colonization in both the study sites. Mycorrhizal structural colonization showed a significant negative correlation ($P < 0.05$) with pH in UTS, whereas in TS, significant negative correlation ($P < 0.05$) with pH was shown by DSE. A list of AMF morphotypes with their relative abundance and isolation frequency is presented in Table 2. *Acaulospora scrobiculata, Glomus macrocarpum and Rhizophagus intraradices* were found to be dominating in both TS and UTS. Shannon (H') and Simpson (D) index were evaluated for the diversity of AMF in *C. sinensis* plantation. H' value was lower in TS (3.24) than that in UTS (3.40). However, D value was higher at TS (0.05) than at UTS (0.04).

A general presence of high AMF colonization levels was observed in all the root samples of *C. sinensis* which is known to be naturally infected by AMF (Diaz and Honrubia, 1993). The sparseness of DSE might be due to its more prevailing condition in extreme environments (Songachan and Kayang, 2011). With increase in growth period after infection, root colonization of host also increased. The AMF colonization could be coordinated with growth stages of plants (Kennedy et al. 2002). The colonization potential was higher in UTS, where the soil had comparatively low P content, which is in accordance with other studies (Galvez et al. 2001). Root colonization percentage as well as spore accumulation showed significant variation in dormant (October–March) and active growth seasons (April–September) as observed by Toman and Jha (2011). Colonization increased significantly during the period of dormancy, in case of both TS and UTS, which was also reported by Singh et al. (2008), who found that percent colonization increased to a significant level during the period of dormancy, in case of natural and cultivated tea bushes, respectively. The lack of correlation between soil P and mycorrhizal parameters agreed with the observations of other researchers (Singh et al. 2003; Songachan and Kayang, 2011; and Priyadharsini et al. 2012). Contrarily, to the observations of Priyadharsini et al. (2012), AMF hyphae showed significant negative correlation with pH in the present study. However, this study is in line with the observations made by Wang et al. (2008) who pointed out that increasing soil pH had detrimental effects on mycorrhization.

Spore density was found to be clearly influenced by seasonal changes. It was recorded higher in dormant period (October–March) than in the active growth period (April–September), which may be due to excess water availability which might have reduced the amount of spores in the soil (García et al. 2008). *Glomus* was the most common genus followed by *Acaulospora* in both the sites, which is consistent with the study of Toman and Jha (2011) who recorded *Glomus* as the dominating genus, followed by *Acaulospora* in the tea rhizosphere of upper Brahmaputra Valley. Our study coincides with the findings of Singh et al. 2008 which proved strong dominance of the genus *Glomus* in cultivated as well as natural tea bushes. This is due to both the high number of described species in these genera as well as their spread and adaptability (Daniell et al. 2001). Also, Glomeraceae are capable of colonizing even with fragments of mycelium (Songachan and Kayang, 2011). Single species each of *Ambispora, Corymbiglomus, Dentiscutata* and *Dominikia* were detected in our study. Very low number of *Gigaspora* might be due to the phenomenon of selection pressure. Agricultural practices may have induced the selection pressure in such a way that specific groups of organisms could establish better than the others. Species of the genus *Glomus* could be considered more tolerant to various practices followed in tea cultivation (Singh et al. 2008). Similarly, regular application of higher doses of nitrogen and phosphorus fertilizer also decreases the number of

large-spored species (Toman and Jha, 2011) such as *Gigaspora.*

CONCLUSIONS

Tea cultivation has become a blooming business providing employment to a large number of people. Small scale tea growers tend to apply sufficient amounts of chemical fertilizers as well as pesticides for high yield, overlooking its adverse impact on soil quality and soil microflora as well. Keeping this in mind, biological means of promoting tea cultivation among the tea growers needs to be encouraged. The present study indicates that tea rhizosphere harbours a plethora of AMF species. Since AMF are naturally present in most soils, their unique fertilizer abilities are already being utilized by most crop plants. However, if suitable methods are developed for cost-effective AMF production and application, it will certainly encourage as well as benefit many small-scale tea cultivators.

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APPENDICES

Table 1: Soil Physico-chemical Properties of C. Sinensis in Untreated Site (UTS) and Treated Site (TS)

Soil Physico-chemical Properties	UTS	TS
pH	5.86 ± 0.06	5.7 ± 0.07
Moisture content (%)	14.67 ± 2.23	16.99 ± 1.91
Temperature (°C)	29.38 ± 1.48	26.75 ± 1.61
Organic carbon (%)	0.96 ± 0.01	1.01 ± 0.01
Available phosphorus (%)	0.013 ± 0.01	0.018 ± 0.02

Table 2: Isolated AMF Species with their Relative Abundance and Isolation Frequency

AMF Species	RA		IF (%)
	UTS	TS	
Acaulospora bireticulata F.M. Rothwell & Trappe	1.03	1.29	100
Acaulospora capsicula Blaszk.	1.53	1.66	100
Acaulospora delicata Walker, Pfeiffer & Bloss	3.57	5.36	100
Acaulospora denticulata Sieverding & Toro	1.94	3.59	100
Acaulospora excavata Ingleby & C. Walker	1.45	3.78	100
Acaulospora foveata Trappe & Janos	3.71	4.70	100
Acaulospora koskei Blaszk.	1.19	—	50
Acaulospora laevis Gerd. & Trappe	0.90	1.66	100
Acaulospora rehmii Sieverd & S. Toro	1.40	1.73	100
Acaulospora scrobiculata Trappe	5.36	6.38	100
Ambispora gerdemannii (S.L. Rose, B.A. Daniels & Trappe) C. Walker *et al.*	1.06	—	50
Claroideoglomus claroideum (N.C. Schenck & G. S. Sm.) Walker & Schuessler	1.36	2.10	100
Claroideoglomus etunicatum (W.N. Becker & Gerd.) C. Walker & Schuessler	3.57	2.35	100
Claroideoglomus luteum (L. J. Kenn, Stutz & Morton) Walker & Schuessler	—	2.44	50
Corymbiglomus globiferum (Koske & Walker) Blaszk. & Chwat	2.86	2.46	100
Dentiscutata heterogama (Nicolson & Gerd.) Sieverd., F. A. Souza & Oehl	1.39	0.41	100
Dominikia aurea (Oehl & Sieverd) Blazsk., Chwat, Silva & Oehl	0.45	1.17	100
Funneliformis badium (Oehl, D. Recker & Sieverd.) C. Walker & Schuessler	1.23	0.60	100
Funneliformis caledonium (Nicolson & Gerd.) Walker & Schuessler	1.53	2.26	100
Funneliformis geosporum (T.H. Nicolson & Gerd.) C. Walker & Schuessler	3.52	2.81	100
Funneliformis mosseae (T.H. Nicolson & Gerd.) C. Walker & Schuessler	3.57	3.73	100
Gigaspora decipiens Hall & Abbott	0.68	—	50
Gigaspora margarita W.N. Becker & I.R. Hall	1.08	0.48	100
Glomus albidum C. Walker & L.H. Rhodes	1.39	0.81	100
Glomus australe (Berk.) S.M. Berch	3.18	4.01	100
Glomus boreale (Thaxt.) Trappe. & Gerd	0.87	0.48	100
Glomus fuegianum (Spegazzini) Trappe & Gerdemann	1.74	1.24	100
Glomus glomerulatum Sieverd.	5.24	5.00	100
Glomus halonatum Rose & Trappe	0.53	—	50
Glomus hoi Berch & Trappe	1.58	0.48	100
Glomus macrocarpum Tul. & Tul.	5.68	5.46	100
Glomus multicaule Gerd. & B.K. Bakshi	1.23	0.55	100
Glomus multisubtensum Mukerji, Bhattacharjee & Tewari	3.42	4.84	100
Glomus reticulatum Bhattacharjee & Mukerji	1.42	0.30	100
Glomus sp. 1	4.05	2.65	100
Glomus tenebrosum (Thaxt.) S.M. Berch	1.69	3.68	100
Racocetra gregaria (N.C. Schenck & T.H. Nicolson) Oehl, F.A. Souza & Sieverd.	—	0.53	50
Racocetra verrucosa (Koske & Walker) Oehl, F. A. Souza & Sieverd.	0.37	—	50

Rhizophagus aggregatus Walker	1.73	1.47	100
Rhizophagus clarus (T.H. Nicolson & N.C. Schenck) C. Walker & Schuessler	1.68	1.87	100
Rhizophagus fasciculatus (Thaxt.) C. Walker & Schuessler	3.19	2.03	100
Rhizophagus intraradices (N.C. Schenck & G.S. Sm.) C. Walker & Schuessler	4.84	6.36	100
Sclerocystis clavispora Trappe	1.40	0.74	100
Sclerocystis sinuosa Gerd. & B.K. Bakshi	1.40	0.51	100
Scutellospora nigra (J.F. Redhead) C. Walker & F.E. Sanders	1.00	—	50
Scutellospora pellucida (Nicol.& Schenck) Walker & Sanders	1.00	0.69	100
Scutellospora reticulata (Koske, D. D. Mill & Walker) C. Walker & F. E. Sanders	0.37	0.18	100
Scutellospora scutata Walker & Dieder	1.26	0.48	100
Septoglomus constrictum (Trappe) Sieverd., G.A. Silva & Oehl	3.52	0.97	100
Septoglomus viscosum (T.H. Nicolson) C. Walker *et al.*	1.94	1.24	100

'—' indicates the absence of a species

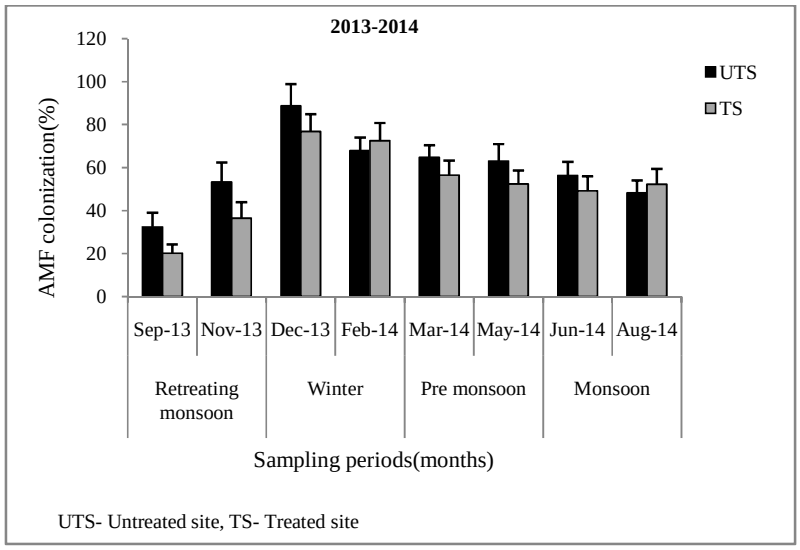

Figure 1: AMF Colonization in C. sinensis

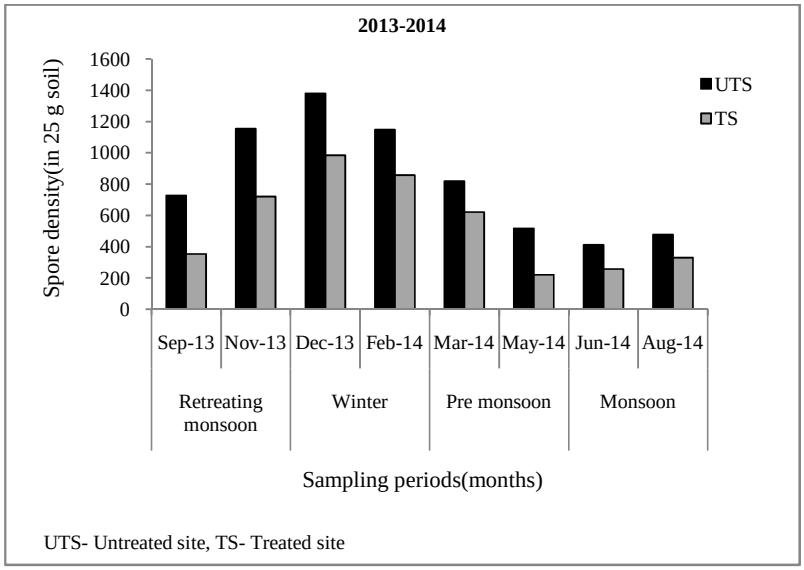

Figure 2: AMF Spore Density of C. sinensis